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Brief Article

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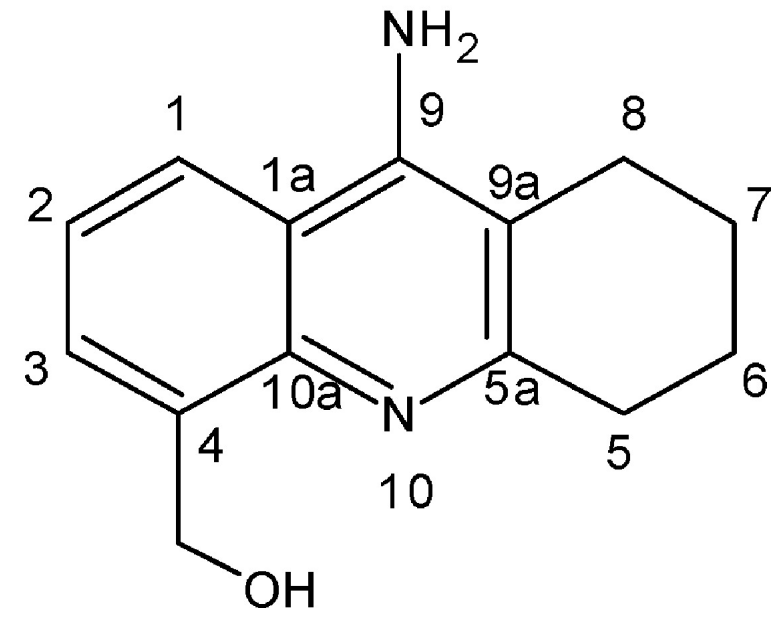
ATAM



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Random Chemistry as a New Tool for the Generation of Small Compound Libraries: Development of a New Acetylcholinesterase Inhibitor

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Random chemistry, the serendipitous generation of small compound libraries by γ-irradiation of source compounds, presents a methodology providing reassembled and rearranged structures. The γ-irradiation was applied to generate new acetylcholinesterase (AChE) inhibitors. The bioassay-guided fractionation as a deconvolution strategy was employed to analyze gained product mixture. The structure of the new highly potent AChE inhibitor, 9-amino-5,6,7,8-tetrahydroacridin-4yl)methanol (**1**), was elucidated by NMR spectroscopy and ESI (tandem) mass spectrometry.

Introduction

At the beginning, drug research relied on the offer of "Mother Nature". Later, the diversity of compounds originating from plants was changed into synthetic diversity produced by organic chemists. After understanding the ligand−receptor interactions, much effort was put into disclosing and defining the target proteins.[1] Structure-based drug design enabled the flexible docking of ligands and de novo design of ligands.[2,3] Since classical syntheses could not cope with the number of compounds needed in the drug discovery process, combinatorial chemistry emerged as a set of novel strategies for the synthesis of large sets of compounds for biological evaluation.[2,4] Despite its lack of diversity, this strategy was recognized as one of the core discovery tools in combination with high-throughput screening.[5]

Here, we report on the random strategy for generation of small libraries[6,7] by means of γ-radiation as an initiator of random free radical recombinations in aqueous or alcohol solution of educts. By exposure of a sample to γ-irradiation, primary and secondary products of solvent radiolysis are generated. These can react with the dissolved compound and can build products.[8,9] The starting compound should be a drugable substance consisting of pharmacophoric elements that we expect to be rearranged through γ-irradiation in a new way. Such a new compound might represent new leads for further drug development.

Acetylcholinesterase (AChE) inhibitors represent the most widely used therapies for neuromuscular diseases and cognitive disorders. Over past 20 years, many drugs have been developed for clinical use in the treatment of Alzheimer's disease.[10−12] Even though their activity was very promising, many of them, e.g., tacrine, showed only limited clinical success because of side effects, Therefore, we searched for new compounds or a lead structure for treatment of Alzheimer's disease. Via random chemistry, we generated a small mixture-based compound library followed by separation, deconvolution, isolation, and characterization of biologically active compounds.

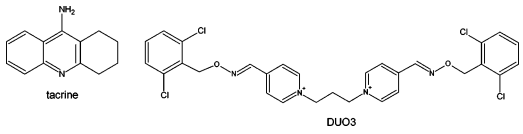

Figure 1. Structural formulas of γ-irradiated compounds.

Results and Discussion

Potent AChE inhibitors tacrine and DUO3 (Figure 1), the latter also known as an allosteric modulator of muscarinic ligand binding,[13] were subjected to γ-rays from a ^{60}Co source. The solvents were selected on the basis of the solubility of substances and with intent to obtain polar hits. The European Pharmacopoeia[14] reports the tacrine hydrochloride to be "very soluble" in water, methanol, and ethanol, "soluble" in propanol, and "sparingly soluble" in ethylene glycol. Hence, tacrine hydrochloride was dissolved in methanol, ethanol, ethylene glycol, and propanol. Owing to the low solubility of tacrine hydrochloride in ethylene glycol, methanol was added to enable complete dissolution. DUO3 was dissolved in DMSO and suspended H_2O.

Upon irradiation, the samples underwent a series of changes. Visual observation of irradiated solutions showed the development of color during irradiation: blue (tacrine/methanol, tacrine/ethylene glycol/methanol), yellow (tacrine/water), green (tacrine/ethanol), and orange (tacrine/propanol).

For the sake of comparison, the starting compound tacrine and all the irradiated samples were initially tested for biological activity by means of Ellman's test[15] *prior* to HPLC fractionation. The obtained IC_{50} values in Figure 3 indicate the inhibition of AChE activity by a certain tacrine solution after irradiation when the part of tacrine was converted. The mixtures were diluted 1:10 and applied to the bioassay in the same way as the nonirradiated control solution of tacrine. The IC_{50} values were assigned as if the reaction mixtures contained

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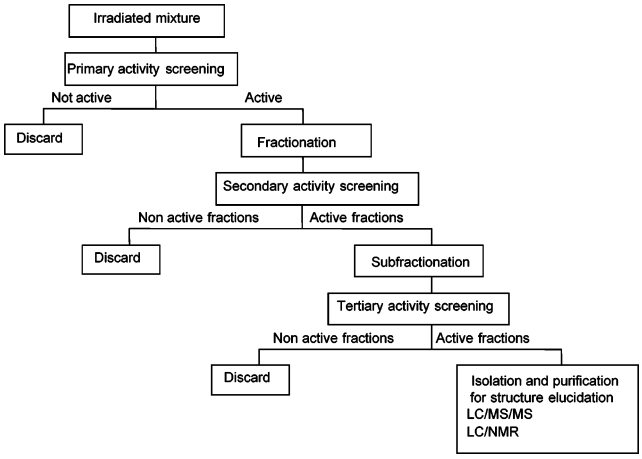

Figure 2. Workflow of the bioassay-guided fractionation steps of irradiated samples and structure elucidation of biologically active compounds.

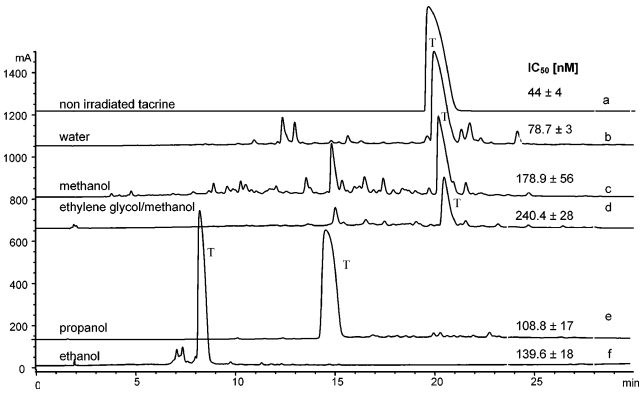

Figure 3. Irradiation process of tacrine depending on the choice of solvent demonstrated by the HPLC results obtained by either irradiation: T = extant tacrine after irradiation; IC_{50} [nM], primary screening of γ-irradiated samples toward AChE. IC_{50} [nM] indicates the concentration of a certain tacrine solution that half-maximally inhibited the AChE activity. Since the irradiated samples represent a multicomponent mixture of unknown composition, the IC_{50} values were computed as if the reaction mixtures contained unchanged tacrine only. HPLC conditions are as described in the Experimental Section. HPLC gradient is as follows: (a−d) A/B (90:10) to 40% B in 30 min; (e) A/B (90:10) to 60% B in 30 min; (f) A/B (80:20) to 70% B in 30 min.

unchanged tacrine only. The general subsequent workflow is depicted in Figure 2.

Primary Activity Screening. Inspection of the HPLC separation reveals the diversity of generated hits in the tacrine/ethanol and tacrine/propanol samples to be poor (Figure 3e,f); nearly the entire amount of tacrine remained intact. The chromatograms (Figure 3c,d) and mass spectra (not shown) of the tacrine/methanol mixture and tacrine/ethylene glycol/methanol indicated the quality of both libraries to be similar; however, the number of members in the ethylene glycol/methanol library is less. The low amount of irradiation products in the tacrine/ethylene glycol/methanol mixture might be a result of the low solubility of tacrine hydrochloride in ethylene glycol, assuming that the small amount of irradiation-generated products arises from the portion dissolved in methanol. Irradiation of tacrine solutions in water and methanol, both being very good solvents for tacrine, gave a higher number of products even though some tacrine remained intact upon irradiation (60% in water, 34% in methanol). In contrast, corresponding ethanol solutions of tacrine do not provide as many products as methanol and water but more than propanol, being a rather poor solvent of tacrine.

An aqueous suspension and a dimethyl sulfoxide (DMSO) solution of DUO3 were also irradiated but did not result in libraries worth studying with regard to biological activity and diversity (data not shown).

Taken together, the choice of the solvent plays a pivotal role in the process of irradiation with the objective of obtaining a compound library. The applied solvent considerably influences the mechanism of the reaction and participates in the chemical reaction. The solubility of the compound in the solvent and the reactivity of the solvent are important.

Fractionation. To find new AChE inhibitors, HPLC fractionation was accompanied by the bioassay. The library was separated into 12 fractions collected by HPLC, which were subjected to a modified Ellman's test[16] to check the inhibitory activity toward AChE. Fractions of samples tacrine/propanol and tacrine/ethanol were found to have lower activity. Consequently, these libraries were dropped from further consideration. Since significant activity was observed for the tacrine/methanol and tacrine/water mixture, they were chosen for further scrutiny. Biological screening of the samples resulting from the first fractionation revealed the tacrine/methanol mixture to be the one with the most single active fractions toward AChE. Thus, the subfractionation and further deconvolution protocol was performed only with these samples.

Tacrine/Methanol. The tacrine/methanol library contained over 50 members. The HPLC fractionation showed some fractions with high inhibitory potential toward AChE. Since fractions F8 (85.6%) and F9 (37.6%) show mass spectra with $m/z = 199$, the activity of these fractions correspond to unchanged tacrine. Fractions F6, F7, and F10 inhibited the enzyme to 26%, 47%, and 24%, respectively. MS analysis revealed most of the fractions to contain more than one compound. Therefore, subfractionation with a tertiary biological screening of highly active fractions was performed applying the same HPLC conditions as described for the first fractionation, but subfractions were taken every 60 s so that each fraction was sliced into three subfractions and tested for biological activity to find out the more exact time position of bioactive substances intended to be isolated. To get a significant response in Ellman's test, the three times pooling of subfractions was carried out. This was necessary to enable the comparison of fractionation (3 mL) and subfractionation sample volume (3 × 1 mL) used for bioassay. Finally, an amount of 100 μL was applied to the bioassay. Figure 4 summarizes the relative inhibitory activities (%) of tertiary biological testing of subfractions 17−20, 28, and 29, exhibiting significant AChE inhibitory effects. The most active subfraction SF20 contained a hit with $m/z = 229$, being also found in SF21. SF18 of F6 contained two species, m/z 231 and 233. SF19 contained a compound with $m/z = 233$, and SF28 and SF29 of F10 shared the same compound with $m/z = 213$.

Structural information of the generated hits was provided by subsequent MS^2 and MS^3 experiments via isolation and fragmentation of a precursor ion. All

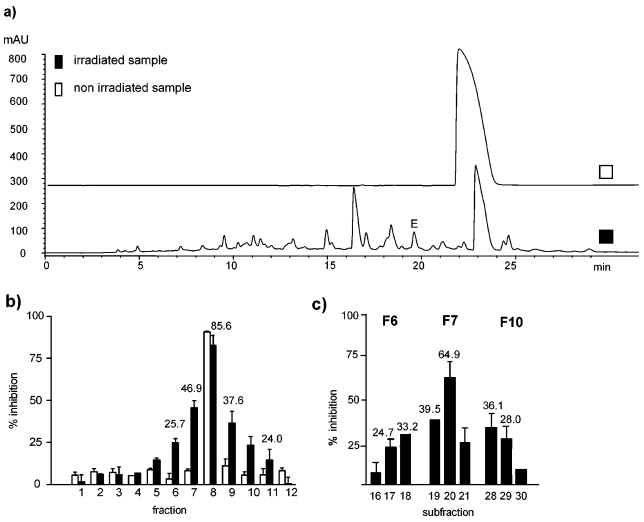

Figure 4. (a) HPLC separation of tacrine/methanol sample. HPLC conditions are as described in Experimental Section. HPLC gradient is as follows: A/B (90:10) 10−35% B in 30 min. (b) Bioassay-guided fractionation of tacrine/MeOH sample. (c) Bioassay-guided subfractionation of this sample. "E" in part a indicates the position of ATAM in chromatogram.

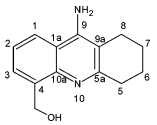

Figure 5. Structural formula of the new potent AChE inhibitor (ATAM, **1**).

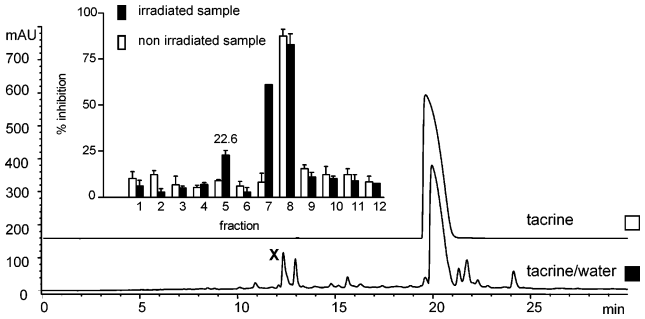

Figure 6. HPLC separation of irradiated tacrine/water sample. "X" indicates the position of 1-hydroxytacrine in the chromatogram. For HPLC conditions, see the Experimental Section. HPLC gradient is as follows: A/B (90:10) to 40% B in 30 min.

compounds showed strong fragmentation in the positive ionization mode. The most inhibitory active compound (peak E, Figure 4a, SF20, SF21) with $m/z = 229$ followed the mass fragmentation pathway to protonated tacrine ($m/z = 199$). Daughter ions observed were at 211 and 199, indicating the removal of water and formaldehyde, respectively, from the protonated molecular ion. The similarity of the UV spectra of tacrine and the substance indicated that this compound, being the most potent product of the tacrine/methanol library, is a tacrine derivative. Further biologically active compounds remained to be studied and will be reported later.

The structure of this hit of the tacrine/methanol library ($m/z = 229$ in SF20) was elucidated. Since it was not possible to isolate enough material for structure characterization in one analytical run, the active compound was isolated by semipreparative HPLC applying the approach of linear scale-up combined with overloading of the chromatographic system. The column length was kept constant (150 mm), the eluent flow was calculated and increased proportionally, and sample volume was adapted correspondingly. It was possible to scale-up the analytical application from a column of 4.6 mm i.d. to one with 10 mm i.d. without any loss of resolution and any method redevelopment. Again, the fraction pooling feature was used to obtain the required amount of active substance. The solid-phase extraction (C18) was used to obtain an up-concentrated amount of compound. Finally 2 mg of product as a yellowish powder was isolated. MS and UV results of the compound were supported by NMR results to confirm the structure named ATAM (=9-*a*mino-5,6,7,8-*t*etrahydro*a*cridin-4yl)*m*ethanol **1** (Figure 5).

The FTIR spectrum showed an OH and an NH band. The mass of the active hit was 228. The ^{13}C NMR spectra and the NMR signals for hydrogens H-5, H-6, H-7, and H-8 indicated an intact tacrine scaffold due to nearly the same chemical shift as in tacrine. The chemical shifts of aromatic hydrogens were different; two doublets at 7.60 and 7.37 ppm and one double doublet at 7.29 ppm were detected, indicating a substitution in position 1 or 4. The isolated CH_2 signal found at 5.10 ppm (^1H NMR) and 65.7 ppm (^{13}C NMR) revealed a neighboring hydroxyl group and an aromatic system. In the HMBC experiment, a long-range coupling to C-10a (145.7 ppm) and two other carbons at 126.5 ppm (C-3), and one was C-4 (137.2 ppm), was observed, indicating that ATAM (**1**) was substituted in position 4 with a CH_2OH group. The purity of **1** was about 91%.

1 inhibited AChE half-maximally at $IC_{50} = 125 \pm 5$ nM, which is in the same range as tacrine ($IC_{50} = 44 \pm 4$ nM) (Figure 6). We expect this novel hydrophilic aminoacridine derivative **1** to metabolize quickly (possibly by glucuronidation or sulfatation) and to have a more favorable clinical profile with fewer side effects compared to tacrine. However, the synthesis of this compound is necessary to study the pharmacology and pharmacokinetics of **1** in more detail. This work is in progress.

Tacrine/Water Sample. Even though the biological activity of the tacrine/water sample was the highest one among irradiated samples ($IC_{50} = 78.71 \pm 3$ nM; Figure 3), the single first fractionation cycle showed that most of the activity could be attributed to intact tacrine (F7, F8). Only fraction 5 (F5) contained significantly higher biological activity (22.6%) (Figure 7). The subfractionation of F5 showed that the inhibitory activity stems from a hit with $m/z = 215$. This mass belongs to the human metabolite of tacrine (hydroxytacrine), which was confirmed by chromatography ($t_R = 12.4$ min) and MS. The fragmentation pathway of 1-hydroxytacrine and our hit X was the same: MS m/z 215, MS^2 m/z 197, MS^3 182, 586.

Reproducibility of the Method. The process of γ-irradiation was proved to be reproducible. Three aliquots of tacrine/methanol samples were separately irradiated, and the same result was obtained regarding quantity and quality of generated library. The IC_{50} values of primary biological screening showed following inhibitory activities: 162.2 ± 40, 183.8 ± 87, and 183 ± 40 nM.

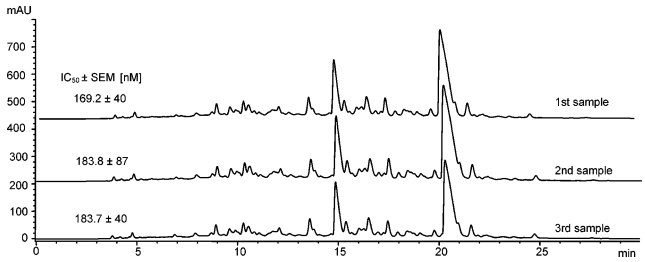

Figure 7. Reproducibility of γ-irradiation process (tacrine/methanol sample). Three independent irradiation procedures were performed, and generated mixtures were analyzed. HPLC separation and biological testing for inhibitory activity toward AChE were conducted. For HPLC conditions, see the Experimental Section. HPLC gradient is as follows: A/B (90:10) to 40% B in 30 min.

Conclusions

Random chemistry proved to be a suitable tool for the generation of small compound libraries. HPLC bioassay-guided fractionation as a deconvolution tool was performed in this study to find compounds of high inhibitory activity toward AChE. MS results showed that radical recombination leads to new compounds in the range of 180−500 mass units. This meets the mass criterion of Lipinski's "rule of five" for druglike molecules.[17] Furthermore, the method showed reproducible results regarding the quality and quantity of members that were in part unknown AChE inhibitors. Further applications to antiinfective drugs in progress show even more interesting libraries.

Experimental Section

^{1}H NMR and ^{13}C NMR and the COSY, DEPT, HMQC, and HMBC results were recorded on a Bruker AV 400 instrument (^{1}H, 400.132 MHz; ^{13}C, 100.613 MHz). IR spectra were obtained using a Biorad PharmalyzIR FTIR spectrometer.

Tacrine and DUO3 were synthesized according to refs 15 and 18, respectively. Solutions of all compounds were prepared in solvents of analytical grade. HPLC and analytical grade methanol were purchased from Merck (Darmstadt, Germany), and ammonium acetate, formic acid, salts, and reagents for the Ellman test were from Fluka (Taufkirchen, Germany). HPLC grade water prepared by means of the Milli-Q purification system (Millipore, Eschborn, Germany) was used throughout.

γ-Irradiation of Samples. Solutions of tacrine hydrochloride (2.34 mg/mL) in five solvents, i.e., water, methanol, ethanol, ethylene glycol/methanol, and propanol, were exposed to γ-rays from a ^{60}Co source, Gammacell 220 Atomic Energy of Canada Ltd. (AECL). Irradiation was performed at PPS Laboratories (Paul Scherrer Institute, Villigen, Switzerland). Solutions were irradiated in glass containers with screw-type plastic caps at room temperature. Air was not evacuated from the containers. The dose rate was maintained at 2.22 kGy/h, and the total dose applied was 500 kGy.

Analytical and Spectroscopic Data for ATAM (1). Formula: $C_{14}H_{16}N_2O$ (228.3). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.60 (1H, d, J = 8.3 Hz, 1-H), 7.37 (1H, d, J = 6.8 Hz, 3-H), 7.29 (1H, dd, J_1 = 8.3 Hz, J_2 = 6.8 Hz, 2-H), 5.10 (2H, s, CH_2-OH), 4.66 (2H, br, NH_2), 3.00 (2H, t, J = 6.0 Hz, 5-H), 2.62 (2H, t, J = 6.1 Hz, 8-H), 2.01−1.92 (4H, m, 7-H, 6-H), 1.20 (br, OH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 157.3 (C-5a) q, 147.0 (C-9) q, 145.7 (C-10a) q, 137.2 (C-4) q, 126.5 (C-3), 123.5 (C-2), 118.9 (C-1), 117.1 (C-1a) q, 110.4 (C-9a) q, 65.7 (CH_2−OH), 34.2 (C-5), 23.6 (C-8), 27.2, 22.7 (C-6, C-7). FTIR, cm^{-1}: 3358 (OH), 3187 (NH_2), 2919, 2850 (CH-valence), 1655, 1581 (C=C-valence). ESI-MS m/z: 229 $[M + H]^+$. MS^2 m/z: 211 (229 $[M + H]^+ - H_2O$), 199 (229 $[M + H]^+ - CH_2O$).

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Supporting Information Available: Details of analytical and preparative HPLC and of Ellman's test. This material is available free of charge via the Internet at http://pubs.acs.org.

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